MgATP-induced inhibition of the adenosine triphosphatase activity of the chloroform-released mitochondrial adenosine triphosphatase

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1. Preincubation of the ox heart chloroform-released mitochondrial ATPase with MgATP results in a time-dependent inhibition of ATPase activity. No re-activation occurs when MgATP remains in the preincubation medium. The enzyme activity returns when all the MgATP in the preincubation system has been hydrolysed. 2. The mechanism of the MgATP-induced inhibition was examined. Inhibition occurs on incubation with MgATP or other hydrolysable nucleotides. Incubation with MgADP or P_i does not cause any inhibition. Neither freshly bound adenine nucleotide nor P_i is associated with inhibited enzyme. The rate of MgATP-induced inhibition correlates with the rate of ATP hydrolysis in the preincubation medium. Changing the rate of ATP hydrolysis at a fixed concentration of ATP also changes the rate of MgATP-induced inhibition by the same proportion. The inhibition is thus related to the ATP-hydrolysis process itself. 3. We propose that intermediate enzyme species of the ATP-hydrolytic sequence can undergo a conformational change to form inhibited species. The kinetics of the inhibition suggest that a substrate-activation step is involved in ATP hydrolysis and MgATP-induced inhibition. 4. The effects of the nature of the preincubation medium on the process of MgATP-induced inhibition and its reversal were examined.

Preincubation of ox heart chloroform-released mitochondrial ATPase (EC 3.6.1.3) with MgATP can result in a time-dependent inhibition of ATPase activity (Lowe *et al.*, 1979b). It has also been shown that F_1 -ATPase (Chernyak *et al.*, 1979) and submitochondrial-particle ATPase (Lowe & Beechey, 1981) are also similarly inhibited by incubation with MgATP.

The inhibitory species involved in the process of MgATP-induced inhibition of ATPase activity was thought to be MgATP, since incubation with MgADP and/or P_i did not result in any inhibition (Lowe *et al.*, 1979b; Lowe & Beechey, 1981). Furthermore, incubation with ATP in the absence of Mg^{2+} or in the presence of EDTA to chelate Mg^{2+} did not result in any inhibition; under these conditions the ATP in the preincubation medium

was not hydrolysed. The inhibition could be caused by the binding of MgATP to either a non-catalytic site or a catalytic site, i.e. it could be related to the process of ATP hydrolysis.

The following observations suggested that the inhibition was due to the interaction of MgATP at a catalytic site. The time course of inhibition induced by preincubation with MgATP paralleled that of ATP hydrolysis, and the extent of inhibition was proportional to the amount of ATP hydrolysed in the preincubation system (Lowe *et al.*, 1979b). Variations in the pH (Lowe *et al.*, 1979b) or temperature of the preincubation medium changed the rate of ATP hydrolysis and the rate of MgATP-induced inhibition by similar factors.

In the present paper we examine further the characteristics of the MgATP-induced inhibition of the ATPase activity of the chloroform-released ATPase and show that under some conditions it can be relieved. We provide evidence that ATP hydrolysis is essential for the observed inhibition, but substrate-depletion or product-inhibition is not the cause of the loss of activity. We propose a model in which intermediate enzyme species of the ATP-

Abbreviations used: NTP, nucleoside triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Tes, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-aminoethanesulphonic acid.

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hydrolytic sequence can undergo a conformational change to produce inhibited species.

Experimental

Preparation of chloroform-released ATPase

Chloroform-released ATPase was prepared from ox heart submitochondrial particles as described in Lowe *et al.* (1979b). The freeze-dried ATPase was dissolved directly in the buffer used. The molecular weight was assumed to be 360 000.

Measurement of ATPase activity

Standard ATPase assay. This assay was as described by Lowe *et al.* (1979b). In this assay method ADP production is coupled to the production of pyruvate, which is determined concurrently by using NADH and lactate dehydrogenase.

Preincubation ATPase assay. ATPase was preincubated with MgATP, MgGTP or MgITP in the presence of an NTP-regenerating system, which consisted of 0.7 mg of pyruvate kinase (Sigma type III)/ml and 71 mM-sodium phosphoenolpyruvate. During the preincubation, NTP hydrolysis results in

the production of a stoichiometric amount of pyruvate. The amount of pyruvate produced was determined from the decrease in A_{340} that occurred on the addition of 20 μ l of preincubation medium to the standard ATPase assay medium. The initial rate of ATP hydrolysis in the preincubation was evaluated from a graph of the amount of pyruvate produced against incubation time. The amount of ATP hydrolysed was used to calculate the number of enzyme turnovers that had occurred, assuming that 1 molecule of ATP is hydrolysed per turnover.

Determination of protein

Protein was determined as described by Peterson (1977), by using the trichloroacetic acid precipitation method. The assay was calibrated with bovine serum albumin. Pentex bovine serum albumin monomer was obtained from Miles Laboratories (Elkhart, IN, U.S.A.).

Determination of nucleotides

Concentrations of ATP were determined by measurements of A_{260} of aqueous solutions at pH 7, assuming an absorption coefficient of $15\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$.

Fig. 1. MgATP-induced inhibition of ATPase activity: effects of varying the concentrations of ATP at (a) low ATPase concentration and (b) high ATPase concentration

ATPase (either 71 or 710 μ g of protein/ml) was incubated for 30 min at 30°C in a buffer consisting of 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$, 5 mM- MgSO_4 , 10% (v/v) methanol, 71 mM-phosphoenolpyruvate and 0.7 mg of pyruvate kinase/ml, pH 8.0. Various concentrations of ATP were then added (∇ , 1.4 μ M; $\blacktriangledown$, 2.8 μ M; $\triangle$, 6.9 μ M; $\square$, 14 μ M; $\blacktriangle$, 28 μ M; $\circ$, 69 μ M; $\blacksquare$, 280 μ M; $\bullet$, 7140 μ M) and the incubation at 30°C was continued. ATPase activity was measured by removal of 20 μ l samples at intervals and diluting them 50-fold into the standard ATPase assay medium. Enzyme activity as a percentage of the control preincubated without ATP is plotted on a logarithmic scale against the incubation time after the addition of ATP. The arrows in Fig. 1(b) mark the time at which the ATP-producing potential of the regenerating systems was exhausted.

Measurement of osmotic strength

This was performed by using a Halbmikro-osmometer.

Materials

[2,8-³H]ATP (tetrasodium salt) was obtained from New England Nuclear (Dreieich, W. Germany). [³²P]P_i and [γ -³²P]ATP (triethylammonium salt) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

ATP was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) (product no. A6144). Identical results were obtained with ATP purchased from Boehringer (Mannheim, W. Germany).

Results

Characteristics of the MgATP-induced inhibition of ATPase activity

Preincubation of the chloroform-released ATPase with MgATP results in a time-dependent inhibition of the ATPase activity (see Fig. 1 and Lowe *et al.*, 1979b). The loss of ATPase activity was measured by dilution of samples from the preincubation medium, containing ATPase and MgATP, into an ATPase assay medium. The same rate of ATP hydrolysis was obtained whether ATP was added immediately (to the assay medium) or after 10 min incubation in the ATPase assay medium. Thus the inhibition is stable to dilution. Since the enzyme activity remains constant during the ATPase assay, a period of 1–10 min, the inhibition is effectively irreversible in the presence of MgATP. With longer assay times, particularly if the amount of ATPase in the assay medium is low, the enzyme activity decreases with time, an effect presumably due to the same process of MgATP-induced inhibition (cf. Akimenko *et al.*, 1972).

The MgATP-induced inhibition of ATPase activity is not due to an irreversible denaturation of the enzyme, since re-activation occurs when the enzyme has hydrolysed all the available ATP in the preincubation medium (see Figs. 1b and 2). Furthermore, polyacrylamide-gel electrophoresis, in the absence or presence of sodium dodecyl sulphate, showed no difference between MgATP-inhibited ATPase and control ATPase (results not shown).

The initial rates of inhibition induced by MgATP are identical whether an ATP-regenerating system is present (the present paper) or absent (Lowe *et al.*, 1979b). Incubation of the ATPase with the regenerating system alone (no ATP added) over a period of 5 h did not result in any inhibition of ATPase activity, measured after dilution. Inclusion of an ATP-regenerating system, however, results in pseudo-first-order kinetics of inhibition (Fig. 1), and the maximum inhibition obtained is greatly increased (cf. results in the present paper with those in Lowe *et*

Fig. 2. MgATP-induced inhibition of ATPase activity: effects of the nature of the preincubation medium

ATPase was dissolved at a protein concentration of 70 μ g/ml in the following media: ■, 50 mM-Hepes/KOH/5 mM-MgSO₄ buffer, pH 8.0; ▲, 50 mM-Hepes/KOH/5 mM-MgSO₄/5 mM-Na₂SO₃ buffer, pH 8.0; ●, 50 mM-Na₂B₄O₇/5 mM-MgSO₄/10% (v/v) methanol buffer, pH 8.0. The buffers also contained 70 mM-phosphoenolpyruvate and 0.7 mg of pyruvate kinase/ml. The reaction mixtures were divided into two and 657 μ M-ATP was added to one of each pair. Incubation was continued at 30°C and ATPase activity was assayed by removal of 20 μ l samples at intervals. The ATPase activity of each incubation with ATP is plotted as a percentage of the value for the control incubation without ATP for each of the three preincubation media. The initial rates of ATP hydrolysis in the preincubation were 17, 31 and 3.2 μ mol/min per mg of protein in the Hepes, Hepes/Na₂SO₃ and Na₂B₄O₇ media respectively. An arrow (†) marks the time when the ATP-producing potential of the ATP-regenerating system was expended.

al., 1979b). We believe that this is because ATP hydrolysis causes MgATP-induced inhibition, and the presence of an ATP-regenerating system allows a constant rate of ATP hydrolysis for a prolonged period. Further evidence for the proposal that ATP hydrolysis causes the MgATP-induced inhibition of ATPase activity was cited in the introduction to the

present paper. We have designed experiments to test this hypothesis, and the results presented below suggest that hydrolysis and inhibition are coupled obligatorily.

Binding of exogenous ATP, ADP and P_i to MgATP-inhibited ATPase

A possible cause of the MgATP-induced inhibition was thought to be binding of either ATP or one or both of the hydrolysis products to the ATPase molecule. To investigate this, ATPase was incubated with either [^3H]ATP or [^{32}P]ATP as follows.

Nucleotide binding. ATPase (0.75 mg of protein/ml) was incubated in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol buffer, pH 8.0, at 30°C with 38 μM -[2,8- ^3H]ATP (294 Ci/mol) with an ATP-regenerating system. The volume was 0.33 ml. After 2 h, 80% inhibition of ATPase activity was achieved. Saturated $(\text{NH}_4)_2\text{SO}_4$ solution (0.75 ml) was added to the incubation mixture at 20°C. After the mixture had been kept in ice for 20 min, the ATPase pellet was collected. About 80% of the residual enzyme activity was recovered in the pellet. The amount of radiolabel associated with the ATPase at this stage was 0.56 mol of ^3H /mol of ATPase. The pellet was redissolved in 0.25 ml of 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol buffer, pH 8.0, and was applied to a column (26.5 cm $\times$ 0.7 cm) of Sephadex G-25 (fine grade), equilibrated and eluted with 50 mM-Tris/ H_2SO_4 /1 mM- K_2SO_4 /1 mM-EDTA buffer, pH 8.0 (Leimgruber & Senior, 1976). ATPase-containing fractions were pooled. The recovery of ATPase activity was 110%. The eluted enzyme was still inhibited by 80% relative to a control, but only 0.03 mol of ^3H was now associated with each mole of ATPase. Thus inhibition does not correlate with binding of ATP or ADP consequent to incubation with ATP. Also, gel filtration did not reverse the process of MgATP-induced inhibition (see the Discussion section).

Phosphate binding. ATPase was preincubated with [γ - ^{32}P]ATP in order to test whether P_i , produced by ATP hydrolysis, could bind to the ATPase and cause the inhibition of enzyme activity. The conditions of incubation differed from those described above for measuring nucleotide binding for two reasons: firstly, an ATP-regenerating system could not be used because [^{32}P]phosphoenolpyruvate was unavailable; secondly, in the absence of an ATP-regenerating system, a relatively low ATPase concentration had to be used in order to get a reasonable inhibition during preincubation with ATP (Lowe *et al.*, 1979b).

ATPase (100 μg of protein/ml) was incubated with 7.4 mM- $[\gamma$ - ^{32}P]ATP (273 c.p.m./pmol) in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol buffer, pH 8.0, at 30°C, in a total volume of 0.2 ml.

After 60 min the enzyme activity was inhibited by 50%. A sample (0.19 ml) of this incubation mixture was applied to a Sephadex G-25 column, as described above. The recovery of enzyme activity was only 57% (large losses of activity occur when dilute enzyme is applied to this column), the eluted enzyme still being inhibited relative to a control. The amount of radiolabel associated with the enzyme was 1.1 mol of ^{32}P /mol of ATPase. This radiolabel must be associated with P_i , since the nucleotide-binding experiments described above show that no adenine remained bound after passage through this column. Re-application of the ATPase to the column separated all radiolabel from the enzyme, but with no detectable increase in enzyme activity.

A control was run to test whether the ^{32}P bound after one passage through the Sephadex column was derived directly from ATP or could have come from P_i released into the medium as a result of ATPase activity. ATPase was incubated with 7.4 mM- $[\gamma$ - ^{32}P]ATP under the same conditions as with [^{32}P]ATP. After a single elution from the column the enzyme was fully active and showed an apparent binding of 0.9 mol of ^{32}P /mol of ATPase. The bound P_i derives from the medium and thus cannot be the cause of the inhibition, since incubation with exogenous P_i causes no inhibition of ATPase activity (Lowe *et al.*, 1979b).

From these experiments it appears unlikely that binding of P_i is the cause of the MgATP-induced inhibition, although a role in the mechanism is not eliminated.

Inhibition of ATPase activity by preincubation with other hydrolysable nucleoside triphosphates

ATPase, at a protein concentration of 71 μg /ml, was incubated with 70 μM -ATP, -GTP or -ITP in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol buffer, pH 8.0, containing an NTP-regenerating system. All three nucleoside triphosphates were hydrolysed, and all three inactivated the ATPase in a process where the rate of loss of enzyme activity is pseudo-first-order with respect to the remaining enzyme activity. The relative rates of hydrolysis of ATP, GTP and ITP in the preincubation were in the proportions 100:6:4 and the relative rates of NTP-induced inhibition were in the proportions 100:13:8. Thus the relative rates of NTP-induced inhibition are similar to the relative rates of NTP hydrolysis (see the Discussion section).

Effect of ATPase activity on the MgATP-induced inhibition

We have shown previously that there is an apparent direct relationship between the MgATP-induced inhibition and ATP hydrolysis (Lowe *et al.*, 1979b). One possible explanation is that both these processes are related solely by their time-

dependence. We consider that this is improbable, since a change of pH from 8.0 to 9.1 decreased the rate of inhibition and the rate of ATP hydrolysis by the same proportion (Lowe *et al.*, 1979b). We performed a further experiment in which the rate of ATP hydrolysis, at constant concentrations of both MgATP and ATPase, was varied by the addition of aurovertin B to the preincubation medium. In the experiment described in the legend to Fig. 3, the half-time for the MgATP-induced inhibition was 28 min in the absence of aurovertin B. In the presence of either 1.2 μ M- or 2.4 μ M-aurovertin B the rate of MgATP-induced inhibition of ATPase activity was diminished by 46% and the initial rate of ATP hydrolysis in the preincubation was diminished by 43%. Thus, at a constant concentration of MgATP, aurovertin B lowers the rate of ATP hydrolysis in the preincubation by the same extent as it lowers the rate of MgATP-induced inhibition.

Fig. 3. *Effects of aurovertin B on the process of MgATP-induced inhibition of ATPase activity*

ATPase at a protein concentration of 71 μ g/ml was incubated for 2 h in a buffer containing 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$, 5 mM- MgSO_4 , 10% (v/v) methanol and 71 mM-phosphoenolpyruvate, pH 8.0, at 30°C. Six samples were taken and incubated at 30°C with no additions, 71 μ M-ATP, 1.2 μ M-aurovertin B, 1.2 μ M-aurovertin B + 71 μ M-ATP, 2.4 μ M-aurovertin B and 2.4 μ M-aurovertin B + 71 μ M-ATP. Samples (20 μ l) were assayed in the standard ATPase assay medium for ATPase activity and for pyruvate production (equivalent to ATP hydrolysed in the preincubation). The inhibition of ATPase activity, expressed as % percentage of the respective incubations with no ATP, is plotted against the amount of ATP that has been hydrolysed in the preincubation medium. ●, 71 μ M-ATP; ▲, 1.2 μ M-aurovertin B + 71 μ M-ATP; ■, 2.4 μ M-aurovertin B + 71 μ M-ATP.

In Fig. 3 it can be seen that the extent of inhibition is directly proportional to the number of enzyme turnovers, irrespective of the time required for the inhibition. This confirms that the processes of MgATP-induced inhibition and ATP hydrolysis are related directly, i.e. that time is not the only common feature.

Effects of the ATP and ATPase concentrations on MgATP-induced inhibition

Two concentrations of ATPase (71 and 710 μ g of protein/ml) were preincubated with various concentrations of ATP, kept constant by an ATP-regenerating system. The loss of enzyme activity with time is shown in Figs. 1(a) and 1(b) for the two ATPase concentrations. At the lower ATPase concentrations (Fig. 1a) the loss of enzyme activity is pseudo-first-order with respect to the remaining enzyme activity for the whole range (1.4–7140 μ M) of ATP concentrations used in the preincubation. Complete inhibition could be obtained with longer incubation times. Increasing the ATP concentration increases the rate of inhibition, but the two are not proportional to each other. The rate of inhibition reaches a maximum value as the ATP concentration is increased. This is shown more clearly in Fig. 4, where the half-time for inhibition is plotted against the reciprocal of the ATP concentration. The plot obtained is not linear for the whole range of ATP concentrations, suggesting that there may be more than one binding site for ATP on the ATPase. Over the range 1.4–69 μ M-ATP, the line is almost straight, giving an apparent K_m of 61 μ M. At higher concentrations of ATP a distinct curvature is observed (Fig. 4 inset), leading to an apparent K_m of >620 μ M. Similar results were obtained at the higher ATPase concentration, with apparent K_m values of 50 μ M and >1500 μ M. The shape of this plot is similar to that of the Lineweaver–Burk plot for ATP hydrolysis by the ATPase, which yields K_m values for ATP at low and high ATP concentrations of 79 and 330 μ M respectively (results not shown). The inference from these results is that ATP hydrolysis is involved in the process of MgATP-induced inhibition.

It can be seen from Fig. 1(b) (high ATPase concentration) that at low ATP concentrations the inhibition increases progressively with time and follows pseudo-first-order kinetics with respect to the remaining enzyme activity. Preincubation with ATP in the concentration range 14–69 μ M-ATP led to total inhibition within the duration of the experiment (7 h). At the highest ATP concentrations (277–7140 μ M), however, a different effect occurs: inhibition increases with time until the ATP-regenerating system has been exhausted. When no more ATP is being hydrolysed in the preincubation medium the inhibition is slowly reversed.

The data listed in Table 1 are derived from the experimental results shown in Fig. 1. It can be seen that at any one concentration of ATP (i) the rate of inhibition does not correlate with the initial rate of ATP hydrolysis in the preincubation. This confirms that the P_i produced during ATP hydrolysis has no

role in the inhibition. (ii) The rate of inhibition correlates with the specific activity of the ATPase measured in the preincubation medium. (iii) To obtain 50% inhibition approximately the same number of turnovers occurs, independent of the ATPase concentration and the rate of hydrolysis of ATP.

The number of turnovers required to achieve 50% inhibition decreases as the ATP concentration in the preincubation medium is lowered. The data in Fig. 5 are the results of an experiment similar to that illustrated in Fig. 1(a). It is clear that at any one ATP concentration the inhibition of the ATPase is proportional to the number of turnovers of the enzyme. The proportionality constant is, however,

Fig. 4. *MgATP-induced inhibition of ATPase activity: kinetics of inhibition at low ATPase concentration*
Experimental results were taken from Fig. 1(a). The half-time for inhibition induced by MgATP is plotted against the reciprocal of the ATP concentration in each incubation. At the higher concentrations of ATP (see inset) a distinct curvature is observed, from which the higher K_m mentioned in the text was calculated.

Fig. 5. *MgATP-induced inhibition of ATPase activity: effects of variations of the ATP concentration in the preincubation medium*

The experimental procedure was as described in the legend to Fig. 1, with an ATPase protein concentration of 71 $\mu\text{g/ml}$ and various concentrations of ATP (O, 7.1 μM ; $\blacktriangle$, 71 μM ; $\blacksquare$, 710 μM ; $\bullet$, 7100 μM). Samples (20 μl) were removed at intervals, and ATPase activity and pyruvate production (stoichiometric with ATP hydrolysis) in the preincubation were determined. From the amount of ATP hydrolysed the number of enzyme turnovers was calculated.

Table 1. *Effects of ATP and ATPase concentrations in the preincubation medium on the MgATP-induced inhibition of ATPase activity*

The data were obtained as described in the legend to Fig. 1. The rate of ATP hydrolysis in the preincubation medium was measured by assaying the pyruvate content of the preincubation medium.

Concn. of ATP in preincubation medium (μM)	$10^3 \times$ Rate of MgATP-induced inhibition (pseudo-first-order rate constants) (min^{-1})		Initial rate of ATP hydrolysis in preincubation medium ($\mu\text{mol/min per ml}$)		Initial ATPase specific activity in preincubation medium ($\mu\text{mol/min per mg of protein}$)		Number of enzyme turnovers required to give 50% inhibition of ATPase activity after dilution	
	71 μg of ATPase protein/ml	710 μg of ATPase protein/ml	71 μg of ATPase protein/ml	710 μg of ATPase protein/ml	71 μg of ATPase protein/ml	710 μg of ATPase protein/ml	71 μg of ATPase protein/ml	710 μg of ATPase protein/ml
690	99	50	0.80	3.8	11.3	5.3	28 200	26 700
69	27	17.3	0.13	0.64	1.8	0.90	17 000	13 000
28	14.1	12.4	0.05	0.36	0.76	0.50	13 400	10 100
14	10.5	7.3	0.03	0.17	0.45	0.24	10 500	8300

dependent on the ATP concentration; at lower ATP concentrations less ATP is hydrolysed to achieve a specified inhibition.

The results in Fig. 1 are interpretable in terms of ATP hydrolysis leading to MgATP-induced inhibition. MgATP at concentrations of $7140\mu\text{M}$ was incubated at a high concentration of ATPase ($710\mu\text{g}$ of protein/ml) (Fig. 1*b*) and at a low ATPase concentration ($71\mu\text{g}$ of protein/ml) (Fig. 1*a*). At the high ATPase concentration maximum inhibition was seen when the ATP-regenerating system was expended. At the low concentration of ATPase the inhibition occurred at a similar rate, but continued until total inhibition was seen. It is thought that the degree of inhibition is related to the number of turnovers of the enzyme. At the high concentration of ATPase each ATPase molecule has undergone 39000 turnovers at the point of maximum inhibition, i.e. when the ATP-regenerating system is expended. From the data in Table 1 and Fig. 5 complete inhibition would be obtained after approx. 50000–60000 turnovers. At the higher concentration of ATPase this number cannot be reached because of the limited capacity of the ATP-regenerating system. In contrast, at the low ATPase concentration, if all the ATP were to be hydrolysed each ATPase molecule would have to undergo 398000 turnovers. This is much greater than the 50000–60000 required to give 100% inhibition. Thus complete inhibition is attained with MgATP still remaining in the preincubation medium.

Fig. 1(*b*) shows that as the concentration of ATP is decreased the rate of inhibition falls, as is to be expected if ATP hydrolysis determines the rate of inhibition. However, the lower the concentration of ATP, the greater the maximum inhibition obtainable. This can be explained if the number of turnovers of the ATPase necessary for the onset of inhibition decreases with decreasing ATP concentrations. That this is the case is shown by the data in Fig. 5.

Effect of the nature of the preincubation medium on the MgATP-induced inhibition of ATPase activity

The inhibitory effects of MgATP on the ATPase are dependent on the preincubation medium. The rate of loss of enzyme activity in $50\text{mM-Na}_2\text{B}_4\text{O}_7$ medium was pseudo-first-order up to high degrees of inhibition (Fig. 2). Complete inhibition occurred before the ATP-regenerating system was exhausted. In contrast, in other media based on 50mM-Hepes/KOH buffer, pH 8.0, or $50\text{mM-Hepes/KOH/5mM-Na}_2\text{SO}_3$ buffer, pH 8.0 (see Fig. 2), or $50\text{mM-Tris/H}_2\text{SO}_4$ buffer, pH 8.0, or 15mM-Tes/Tris buffer, pH 6.7, or lower concentrations of $\text{Na}_2\text{B}_4\text{O}_7$, pH 8.0 (results not shown), the loss of enzyme activity did not follow pseudo-first-order kinetics. The rate of inhibition decreased with time until the ATP in the

preincubation medium had been hydrolysed, when a re-activation occurred.

In the media based on Hepes, Hepes/ Na_2SO_3 and Tes/Tris the initial rates of ATP hydrolysis in the preincubation medium were in the proportions 1:1.8:0.7 and the rates of MgATP-induced inhibition were in the proportions 1:2.5:0.7 (Fig. 2). Thus the rates of MgATP-induced inhibition are again related to the rates of ATP hydrolysis in the preincubation.

The rate of ATP hydrolysis in $50\text{mM-Na}_2\text{B}_4\text{O}_7/5\text{mM-MgSO}_4/10\%$ (v/v) methanol buffer, pH 8.0, was only 19% of that in the Hepes-based medium, but the rate of MgATP-induced inhibition was 1.9-fold greater in the former medium than in the latter (Fig. 2). Thus in borate buffer about 18500 turnovers of each ATPase molecule occur in order to achieve 50% inhibition, whereas in Hepes buffer about 134000 turnovers are necessary to achieve the same inhibition. The difference between the process of MgATP-induced inhibition in borate-based and Hepes-based media is not thought to be due to a difference in buffer concentration. The concentration of Hepes/KOH was varied between 25 and 200mM in the preincubation medium; the measured osmotic strength varied between 55 and 440mosM, but no effects were noted on the MgATP-induced inhibition of ATPase activity. However, changing the concentration of $\text{Na}_2\text{B}_4\text{O}_7$ from 5 to 50mM varied the osmotic strength from 29 to 290mosM and decreased the number of enzyme turnovers required to achieve 50% inhibition of ATPase activity from 57000 to 17000.

It appears that $\text{Na}_2\text{B}_4\text{O}_7$ has a stabilizing effect on the MgATP-inhibited ATPase, since less ATP is needed to be hydrolysed in order to achieve a specified degree of inhibition, and the resultant inhibited enzyme does not revert to active enzyme as rapidly once the MgATP has been hydrolysed. Borate is able to complex with the vicinal diol group of ATP, and it is possible that the specific behaviour of borate in promoting the process of MgATP-induced inhibition is related to this phenomenon.

Discussion

ATP and ADP are known inhibitors of the mitochondrial ATPase activity (Pedersen, 1975; Harris *et al.*, 1978). We believe that the MgATP-induced inhibition of ATPase activity described in the present paper is different.

ADP can act rapidly as a reversible inhibitor of the ATP-hydrolysis activity of the soluble mitochondrial ATPase (Pullman *et al.*, 1960; Hammes & Hilborn, 1971; Pedersen, 1975). The inhibition induced by MgATP described in the present paper is not due to the accumulation of ADP, since the inhibition is not rapidly reversible and incubation of

the ATPase with MgATP and an ATP-regenerating system causes a time-dependent inhibition of ATPase activity, although the concentration of ADP is maintained low and constant. It is expected, however, that in the preincubation medium ADP does act as a reversible inhibitor, but this would not result in an inhibition of the ATPase activity as measured after dilution into the standard ATPase assay.

It has been demonstrated that preincubation of the F_1 -ATPase with ADP can result in a time-dependent inhibition of ATPase activity that is stable to dilution (Gomez-Fernandez & Harris, 1978; Harris *et al.*, 1978; Minkov *et al.*, 1979). The inhibition could be reversed by addition of ATP, and the maximum inhibition attained was only about 60%. Incubation of F_1 -ATPase with MgATP resulted in the same degree of inhibition as occurred on incubation with the same concentration of MgADP (Gomez-Fernandez & Harris, 1978). The inhibition induced by MgATP was thus attributed to the action of the hydrolysis product, MgADP.

The MgATP-induced inhibition of the chloroform-released ATPase activity described in the present paper differs from the inhibition of F_1 -ATPase described by Harris and co-workers in that incubation with MgADP did not result in any inhibition of ATPase activity and complete inhibition could be obtained. We believe that this difference between the F_1 -ATPase and chloroform-released ATPase preparations could reflect a difference in the interactions between endogenous inhibitor protein and the ATPase molecule (Lowe *et al.*, 1979a).

In the present paper and in Lowe *et al.* (1979b) we have presented experimental results that are relevant to an understanding of the mechanism of MgATP-induced inhibition. Direct single-step ATP-binding at a non-catalytic site can be excluded, since the kinetics of inhibition display saturation kinetics and ATP is not found to be bound to inhibited enzyme. Binding of ADP and/or P_i does not cause the inhibition, since accumulation of these products did

not lead to inhibition, and inhibited enzyme did not contain newly bound nucleotide or phosphate. Alternative explanations for the mechanism of the inhibition are thus (a) ATP-binding at a non-catalytic site inducing a change in inhibited enzyme or (b) inhibition consequent to the ATP-hydrolysis process itself, i.e. due to an interaction of ATP at a catalytic site. Evidence in favour of this latter process is: (1) the inhibition occurs in the presence of a hydrolysable nucleotide and the process is Mg^{2+} -dependent; (2) variation of the rate of ATP hydrolysis by the inhibitor aurovertin B, at a constant concentration of MgATP, alters the rate of inhibition to the same extent (Fig. 3); (3) variation of the rate of ATP hydrolysis by changing the temperature or the pH, at a constant concentration of MgATP, alters the rate of inhibition to the same extent (Lowe *et al.*, 1979b); (4) the extent of inhibition is directly proportional to the amount of ATP hydrolysed by the enzyme (Fig. 3); (5) the kinetics of inhibition by MgATP (Fig. 4) display a similar saturation behaviour with increasing ATP concentration as does the rate of ATP hydrolysis. For mechanism (a) to be correct one would therefore have to invoke an unlikely combination of circumstances.

In Scheme 1 is presented a mechanism for the observed inhibition. This scheme is a minimal model that fits the experimental findings. Other more complex models cannot be excluded. Mg^{2+} is essential for both hydrolysis and inhibition, but its exact role is unknown, and therefore it has not been included in the model. In Scheme 1 inhibited enzyme, E^* , is derived from intermediates of the ATP-hydrolysis pathway [$E \cdot ATP$ and $E \cdot (ATP)_2$]. During ATP hydrolysis, $E \cdot ATP$ and $E \cdot (ATP)_2$ are formed. They can either decompose to yield the products of ATP hydrolysis or they can form E^* , with no enzyme activity. E^* is thought to be able to revert to active enzyme in a slow process that is not shown in Scheme 1.

Assuming that (a) E , ATP , $E \cdot ATP$ and

Scheme 1

$E \cdot (ATP)_2$ are all at their equilibrium concentrations and (b) the rate of re-activation of E^* is negligible:

$$\ln [\text{ATPase activity} (\%)] = \frac{K_2 k_{+5} [\text{ATP}] + k_{+6} [\text{ATP}]^2 \cdot t}{K_1 K_2 + K_2 [\text{ATP}] + [\text{ATP}]^2}$$

This equation is consistent with the data in Fig. 1(a), where pseudo-first-order plots for inactivation are observed and the apparent rate constants are dependent on the concentration of ATP in a complex manner (Fig. 4).

It also follows from Scheme 1 that the rate of formation of hydrolysis products divided by the rate of formation of inhibited enzyme, which is also equal to the number of turnovers of the ATPase needed to produce 1 mol of E^* , is equal to:

$$\left(\frac{k_{+4} [\text{ATP}]}{k_{+3} + \frac{k_{+4} [\text{ATP}]}{K_2}} \right) \quad (1)$$

$$\left(\frac{k_{+6} [\text{ATP}]}{k_{+5} + \frac{k_{+6} [\text{ATP}]}{K_2}} \right)$$

Eqn. (1) would predict that, at any one concentration of ATP, the extent of the inhibition of ATPase activity is directly proportional to the number of turnovers of the ATPase molecule. In general this is seen (Figs. 3 and 5), although as the concentration of E^* becomes high a deviation from proportionality is observed (Fig. 5). As E^* accumulates its conversion into active enzyme becomes significant, and hence relatively more turnovers are needed to achieve further inhibition. Eqn. (1) further predicts that the slope of the plot of inhibition against number of turnovers (Fig. 5) would be a function of the concentration of ATP, with a maximum slope (k_{+4}/k_{+6}) at high ATP concentrations and a minimum slope (k_{+3}/k_{+5}) at low ATP concentrations. This is indeed observed. The data from Table 1 are plotted in Fig. 6. In agreement with the predictions of Scheme 1, a straight line is obtained, of gradient 0.96 (predicted gradient = 1).

We believe that MgATP-induced inhibition observed in Hepes buffer (Fig. 2) also follows the mechanism shown in Scheme 1. Differences in the kinetics of inhibition between those observed in Hepes-based and borate-based preincubation media are due to different values for the rate constants. The small differences in the ratios of the rates of hydrolysis to rates of inhibition of different nucleotides could be explained similarly.

Scheme 1 suggests an explanation for the curvature observed in Lineweaver-Burk plots for ATP hydrolysis by the ATPase and the double-reciprocal plot of MgATP-induced inhibition (Fig. 4), since a substrate-activation step can lead to this shape of plot (Segel, 1975).

Fig. 6. Test of fit of experimental results to Scheme 1. It can be shown that, according to Scheme 1:

$$\log \left(\frac{T - T_0}{T_\infty - T} \right) = \log [\text{ATP}] + \log \left(\frac{k_{+6}}{K_2 k_{+5}} \right)$$

where T is equal to the number of turnovers of the ATPase needed to produce 1 mol of inhibited enzyme at a specified concentration of ATP, T_0 is that number at zero ATP concentration, and T_∞ is that number at an infinite concentration of ATP. In this Figure data from Table 1 ($71 \mu\text{g}$ of ATPase protein/ml) are plotted in order to test whether this equation, and hence Scheme 1, describes the experimental data. T_0 and T_∞ are assumed to be 8000 and 32000 respectively.

Reversal of the inhibition induced by MgATP occurs continuously, but is only noted when its rate is significant compared with that of the inhibitory process. Reversal is seen under circumstances when ATP in the preincubation medium has been hydrolysed and the products of ATP hydrolysis are still present. However, if the inhibited enzyme is separated from products of hydrolysis by gel filtration, reversal is not observed. Thus it would seem that one or a combination of ADP, P_i and Mg^{2+} are necessary for reversal of the inhibition.

We believe that the change from E to E^* involves a conformational change in the ATPase molecule or, more specifically, a change in the interactions between endogenous inhibitor protein and the ATPase molecule (Lowe *et al.*, 1979b, and unpublished work).

A similar inhibition of ATPase activity to that described in the present paper has been observed by Akimenko *et al.* (1972) and by Chernyak *et al.* (1979) with F_1 -type ATPase preparations. Chernyak *et al.* (1979) suggested that the inhibition was due to

a conformational change in the enzyme consequent to ATP hydrolysis, whereas Akimenko *et al.* (1972) proposed that the endogenous inhibitor protein was involved in the process. Their proposals are thus similar to the conclusions presented above.

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